

# Use of 16S rRNA Gene Profiling by Terminal Restriction Fragment Length Polymorphism Analysis To Compare Bacterial Communities in Sputum and Mouthwash Samples from Patients with Cystic Fibrosis†

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**The bacterial communities present in the oral cavity and the lungs of 19 adult cystic fibrosis (CF) patients were compared by using terminal restriction fragment length polymorphism analysis of 16S rRNA gene PCR products amplified from nucleic acids extracted directly from bacteria in clinical samples. Sputum samples were not found to be subject to profound contamination by oral cavity bacteria. Evidence of colonization of the CF lung by certain oral bacterial species was found.**

Traditionally, the detection of bacterial species within the cystic fibrosis (CF) lung has relied on identification through the in vitro cultivation of the microbes within expectorated sputum. By avoiding in vitro culture, however, the recent application of molecular biological approaches has led to the detection of many anaerobic species and many species novel to CF in sputum samples (4, 5). This is because no assumptions are made a priori about what species are present. These molecular approaches obviate the requirement for culture prior to analysis as they amplify bacterium-specific ribosomal gene PCR products from nucleic acids extracted directly from bacteria present in clinical samples. The mixed pool of phylogenetically informative ribosomal genes is then resolved as a set of distinct bands corresponding to the different bacterial species present in the sample by using terminal restriction fragment length polymorphism (T-RFLP) profiling.

Previously, a number of species normally associated with the oropharyngeal environment were identified in CF sputa (4, 5). In these studies, the sputum bolus underwent a series of washing steps to remove bacteria acquired in transit before DNA extraction. While expectorated sputum is often used to indicate bacterial infection, given that it transits through the upper airways and in particular the oral cavity, it is possible that these precautions were not entirely effective. Sampling in parallel the oral environment therefore becomes important to study this. Mouthwash samples have been shown to provide an ideal means of doing so as they provide a cross section of different oral bacterial communities (7).

In this study, we compare the bacterial communities present

in expectorated sputum samples with those present in the corresponding mouthwash sample generated by the same adult CF patient, allowing the level of profile similarity and consequent likelihood of contamination between the CF sputa and oral environment to be determined.

Sputum and mouthwash samples were obtained from 19 adult CF patients attending routine checkups at Southampton University Hospital. All the patients were clinically stable at the time of sampling. None had experienced an infective exacerbation for more than 2 weeks prior to sampling. Patients were asked to rinse their mouth thoroughly using 10 ml of sterile saline (0.9% NaCl, room temperature), with the washings being collected into a sterile container. Thirty minutes later, patients produced spontaneous sputum sample by coughing into a fresh sterile container. Prior to DNA extraction, sputum samples were washed in sodium phosphate buffer to remove adherent saliva, with DNA extraction being carried out as described previously (4). Mouthwash samples were spun at  $7,000 \times g$  for 5 min to concentrate bacterial cells into a pellet, with DNA being extracted as for sputum samples.

The oligonucleotide primers used to amplify a region of the 16S rRNA gene for members of the domain *Bacteria*, 8f700 (5'-IRD700-AGA GTT TGA TCC TGG CTC AG-3') and 926r (5'-CCG TCA ATT CAT TTG AGT TT-3'), were described previously (1). PCRs, amplicon digestion, and T-RFLP profiling were performed as described previously (5).

T-RFLP profiles were analyzed using Phoretix 1D Advanced software v.5.10 (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom), with band sizes determined by comparison to size markers (Microzone, Lewes, United Kingdom). Band volume (the product of the area over which a band was detected and the intensity of signal recorded over that area) was determined and expressed as a percentage of the total volume of bands in a given electrophoretic profile. A threshold of 0.01% of the total profile signal was set as the detection thresh-

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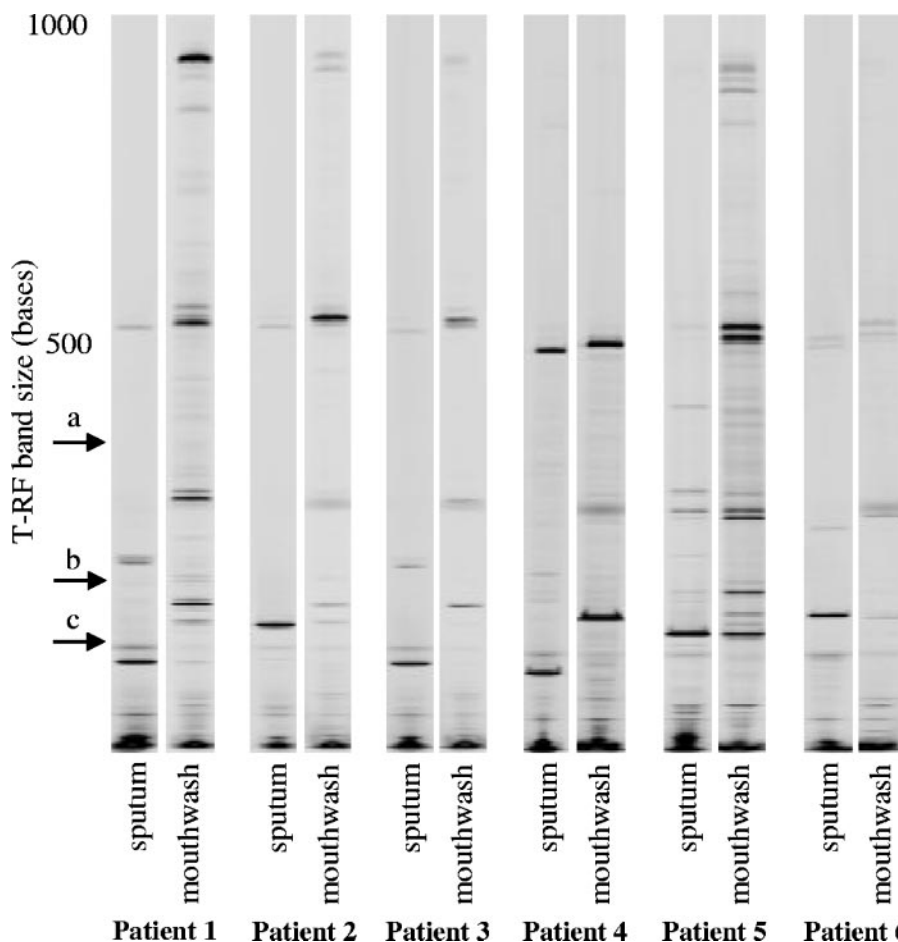


FIG. 1. T-RFLP profiles generated from sputum and mouthwash samples taken from six patients in the sample group. Arrows labeled a, b, and c correspond to the position of bands generated for *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, respectively.

old. Hierarchical cluster analysis using the Dice measure and *t* test calculations were carried out using SPSS for Windows v.10.1 (SPSS Inc., Chicago, Ill.) (6).

Examples of 6 of the 19 paired sets of T-RFLP profiles, selected arbitrarily, are shown in Fig. 1. The mean number of different T-RF band lengths detected in sputum and mouthwash profiles was 14.7 ( $\pm 8.8$ , standard deviation) and 14.2 ( $\pm 10.7$ ), respectively (ranging from 2 to 31 and 3 to 38, respectively). This difference was not significant ( $P > 0.05$  as calculated by *t* test). In eight patients, more T-RF bands were detected in profiles generated from sputa than in those from mouthwash samples. In 10 patients, more T-RF bands were detected in the profiles generated from mouthwash samples than in those from sputum samples, and for one patient, the number of T-RF bands detected in each of the two sample types was the same.

The total number of T-RF bands detected in the sputum and mouthwash samples was 217 and 147, respectively. Band volumes ranged from 0.01 to 92.5% of the total band volume, with a mean of 6.9% ( $\pm 14.4\%$ ). Of the bands detected in mouthwash samples, 91.2% (134) were not detected in any of the sputa. By comparison, 29.5% (64) of the bands detected in the sputum samples were not detected in any of the mouthwash samples. Of the bands detected in sputum and mouthwash,

72.7% ( $\pm 22.0\%$ ) and 74.0% ( $\pm 24.5\%$ ), respectively, were not detected in the corresponding mouthwash and sputum sample from that patient. Of the total number of bands detected in a patient, 10.3% ( $\pm 7.5\%$ ) were detected in both sample types. In 59.1% of cases, the relative volume of such bands was higher in the sputum sample profile.

There were 60 instances in which a band was resolved in a mouthwash sample representing  $>10\%$  of the total band volume (with a mean volume of 26.2% [ $\pm 15.2\%$ ]). In 75% of instances, there was no corresponding band detected in the sputum sample. Of the 15 instances where there was a corresponding band in the sputum profile, in 12 instances the volume of the band in the sputum profile was lower (with a relative volume ranging from 0.8 to 83.0% of that in the mouthwash sample), in two instances it was higher (636.6% and 147.5%), and in one instance it was the same (100.7%). Thirty-seven other bands were detected in sputum profiles that represent more than 10% of total band volume. In 28 cases, no corresponding band was detected in the mouthwash sample. Here the mean band volume was 30.0% ( $\pm 22.8\%$ ). In the remaining nine instances, the volume of the corresponding band in the mouthwash sample had a mean value of 8.0% ( $\pm 14.8\%$ ).

These data were also examined for possible evidence of

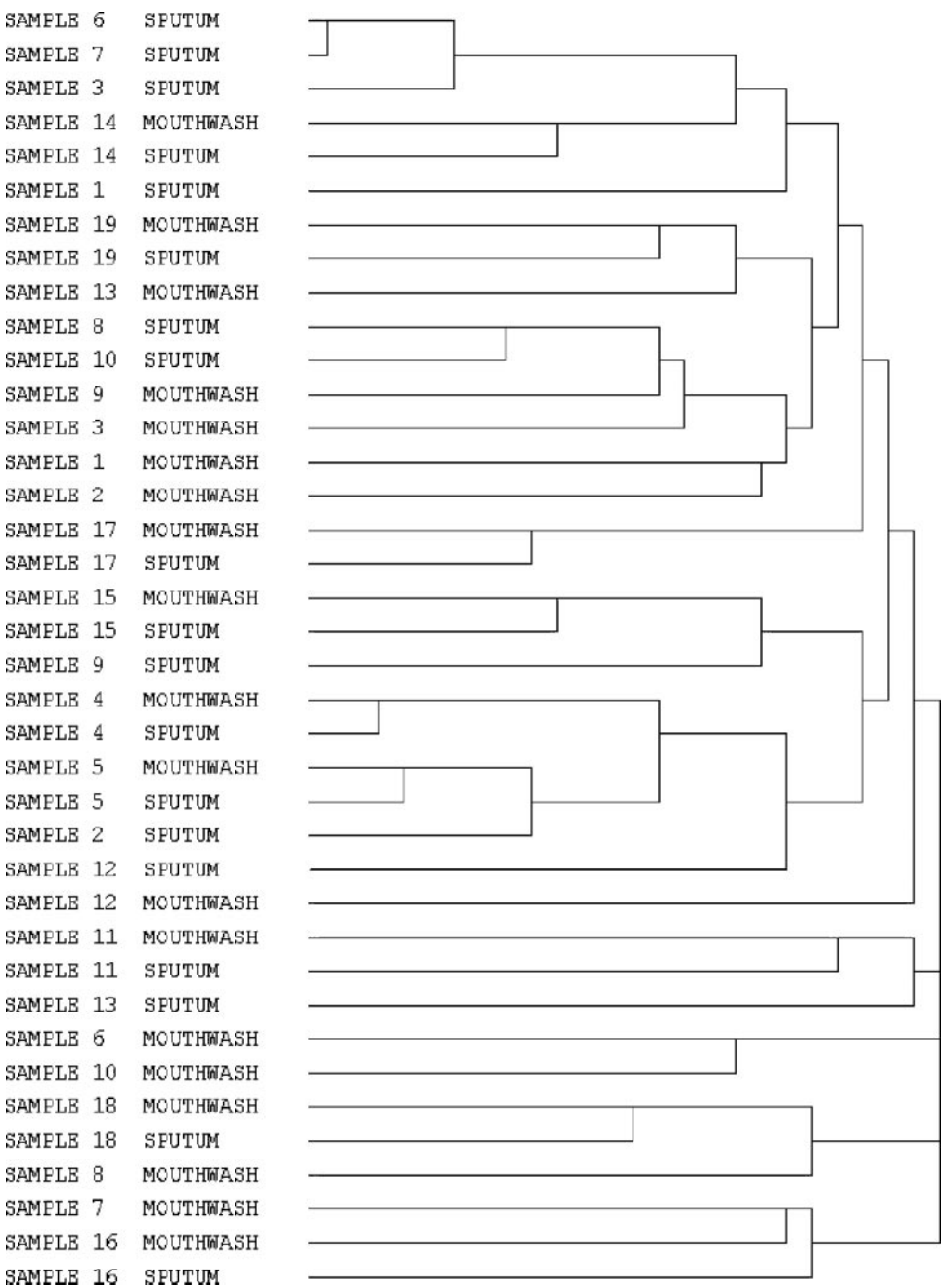


FIG. 2. Dendrogram constructed using hierarchical cluster analysis (Dice measure) showing the relative similarities of T-RFLP profiles generated from the sample set.

“communication” between the bacterial communities that exist in the oral cavity and the CF lung. This was done by determining the degree of similarity of profiles using hierarchical cluster analysis (Dice similarity measure) (Fig. 2). Sixteen samples (eight patients; patients 4, 5, 11, 14, 15, 17, 18, and 19) clustered most closely with the corresponding sample from the same patient. Eleven samples clustered most closely with a sample of the same type (mouthwash samples from patients 1, 2, 6, 7, 10, and 16 and sputum samples from patients 3, 6, 7, 8, and 10). Two samples clustered most closely with the opposite

sample type but from another patient, and nine samples clustered most closely with a mixed cluster.

This study addressed two important questions. Firstly, to what degree is spontaneously expectorated sputum subject to contamination by bacteria present in the upper airways and in particular the oral cavity? Secondly, is there evidence that bacterial species previously associated most strongly with the oral cavity are also present in the lungs of CF patients?

These data address the issue of sample contamination in a number of ways. For samples taken from nine patients, the

band with the highest relative volume in the sputum profile was also detected in the mouthwash profile. In these cases, it could be argued that the presence of the lung-colonizing species in the oral environment may be due to contamination of the mouth during clearance of sputum from the lung. However, more generally, these data provide strong evidence that sputum expectorated from the lungs of CF patients is not contaminated to a significant degree by bacteria present in the oral cavity. If contamination by bacterial species (from either oral cavity to sputum or vice versa) had been significant, then no marked difference would have been apparent in the T-RFLP profiles generated from the same individual. That was not found here. As such, this alone provides support for the contention that CF sputum is not contaminated to a significant extent by bacterial species within the oral cavity. Further evidence for the lack of overt contamination was provided when assessing the relative volumes of the T-RF bands. When the relative volumes of the T-RF bands detected were compared, in 13 of the 19 patients the highest relative volume band in the mouthwash sample was not detected in the corresponding sputum T-RFLP profile. Furthermore, for only two of the remaining six patients did the band of highest relative volume in the mouthwash sample set match that for the sputum sample set. This again suggests that contamination was unlikely. This finding is important as it validates the continued use of sputa in the context of nonselective, molecular biology-based investigative techniques for microbiological analyses as described here.

T-RFLP profiling also enabled comparisons to be made of bacterial community structures in mouthwash and sputum samples from adult CF patients. The oral environment contains bacterial communities of high complexity (3, 7). The data presented here indicate that the number of bacterial species was approximately equal for the two sample types—no statistically significant difference between the two environments sampled was determined. These findings provide further evidence that the bacterial community in the adult CF lung is, in fact, highly diverse (5, 6). Data from this study also indicate that the bacterial community present in the mouthwashes and sputum showed similar species evenness as well as species richness distributions.

The data presented here also suggest communication between the oral environment and the lower airways. Approximately 1 species in every 10 detected in the CF lung matched a band also present in the oral cavity. Further, there was evidence of significant colonization by species as indicated by the presence of multiple, matched T-RF positions in both lung

and oral samples. Moreover, support for this linkage between the two environments was provided by the results of the hierarchical cluster analysis, with the paired profiles from 8 of the 19 patients clustering more closely together than with any of the other samples analyzed.

It is also quite possible that the oral cavity acts as a “stepping-stone” for lung colonization and, in turn, infection for certain bacterial species. Some evidence for this is suggested by T-RF band size uniqueness data: only ca. 10% (134/147) of oral cavity-derived T-RF sizes were specific to the oral cavity, whereas ca. 30% (64/217) of sputum-derived T-RF sizes were specific to the lung samples. These findings are consistent with previous studies suggesting that the teeth can serve as a reservoir for respiratory infection (2, 8, 9).

Overall, therefore, while these findings suggest that oral bacteria may be important colonizing species, much remains to be determined about the process of respiratory disease in CF patients. These findings also underline the potential that such molecular methodologies have for providing fresh insight into lung infections.

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